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The role of estrogen and progesterone, administered alone and in combination, in modulating cytokine concentration following traumatic brain injury

Mohammad Khaksari, Zahra Soltani, Nader Shahrokhi, Gholamreza Moshtaghi, and Gholamreza Asadikaram

Abstract: Cytokines play an important role in the pathophysiology of traumatic brain injury (TBI). This study was designed to determine the effects of administering progesterone (P) and estrogen (E), alone and in combination, on brain water content, blood–brain barrier (BBB) disturbance, and brain level of cytokines following diffuse TBI. Ovariectomized rats were divided into 9 groups, treated with vehicle, E1, E2, P1, P2, E1+P1, E1+P2, E2+P1, and E2+P2. Levels of BBB disruption (5 h), cytokines, and water content (24 h) were evaluated after TBI induced by the Marmarou method. Physiological (E1 and P1) and pharmacological (E2 and P2) doses of estrogen and progesterone were administered 30 min after TBI. Water content in the E1+P2-treated group was higher than in the E1-treated group. The inhibitory effect of E2 on water content was reduced by adding progesterone. The inhibitory effect of E1 and E2 on Evans blue content was reduced by treatment with E1+P1 and E2+P2, respectively. The brain level of IL-1β was reduced in E1 and E2, after TBI. In the E2+P2-treated group, this level was higher than in the E2-treated group. The brain level of TGF-β was also elevated by the administration of progesterone and estrogen alone, and reduced when the hormones were administered in combination. In conclusion, a combined administration of progesterone and estrogen inhibited the decreasing effects of administration of progesterone and estrogen alone on water content and BBB disruption that mediated to change the proinflammatory cytokines.

Key words: estrogen, progesterone, traumatic brain injury, blood–brain barrier, water content, cytokines.

Résumé : Les cytokines jouent un rôle important dans la pathophysiologie de la lésion cérébrale traumatique (TCC). La présente étude a eu pour but de déterminer les effets de l'administration de progestérone (P) ou d'œstrogène (E), séparément ou en association, sur la teneur en eau, l'altération de la barrière hémato-encéphalique (BHE) et le taux de cytokines dans le cerveau après une TCC diffuse. On a divisé des rats ovariectomisés (OVX) en 9 groupes : véhicule, E1, E2, P1, P2, E1+P1, E1+P2, E2+P1 et E2+P2. On a évalué les taux d'altération de la BHE (5 h), les taux de cytokines et la teneur en eau (24 h) après une TCC induite par la méthode Marmarou. On a administré des doses physiologiques (E1 et P1) et pharmacologiques (E2 et P2) d'œstrogène et de progestérone 30 min après la TCC. La teneur en eau a été plus élevée chez E1+P2 que chez E1. L'effet inhibiteur de E2 sur la teneur en eau a été atténué par l'ajout de progestérone. L'effet inhibiteur de E1 et de E2 sur la teneur en bleu d'Évans a été diminué par E1+P1 et E2+P2 respectivement. Le taux cérébral d'IL-1β a diminué chez E1 et E2 après la TCC. Ce taux a été plus élevé chez E2+P2 que chez E2. Le taux cérébral de TGB-β a aussi été augmenté par l'administration séparée de progestérone et d'œstrogène, et diminué par l'administration combinée des hormones. Ainsi, l'administration combinée de progestérone et d'œstrogène a inhibé les effets décroissants de l'administration séparée de progestérone et d'œstrogène sur la teneur en eau et l'altération de la BHE, et conduit aux modifications des cytokines proinflammatoires.

Mots-clés : œstrogène, progésterone, lésion cérébrale traumatique, barrière hémato-encéphalique, teneur en eau, cytokines.

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Abbreviations: TBI, traumatic brain injury; BBB, blood–brain barrier; OVX, ovariectomized; IL, interleukin; TGF-β, transforming growth factor-β.

M. Khaksari.[1] Neuroscience Research Center, Kerman University of Medical Sciences, Kerman, Iran; Bam International Unit, Kerman University of Medical Sciences, Kerman, Iran.
Z. Soltani. Neuroscience Research Center, Kerman University of Medical Sciences, Kerman, Iran.
N. Shahrokhi. Physiology Research Center, Kerman University of Medical Sciences, Kerman, Iran.
G. Moshtaghi. Department of Biochemistry, Kerman University of Medical Sciences, Kerman, Iran.
G. Asadikaram. Department of Biochemistry, Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

[1]Corresponding author (e-mail: khaksar38@yahoo.co.uk).

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Introduction

In postmenopausal women, the incidence of stroke rapidly increases (Wenger et al. 1993), coincident with diminished circulating levels of estrogen and progesterone. Estrogens function as neurotrophic molecules and prevent neuronal cell death under certain conditions. Recent evidence suggests that progesterone may also contribute to these events, albeit to a lesser extent (Hoffman et al. 2006).

Brain trauma produces edema, primary death of neurons at the site of impact and secondary neuronal damage to the underlying area. Focusing on the role of gonadal steroids in brain trauma began with the observation that following traumatic injury, females showed less edema than males (Roof et al. 1993). The investigators then concentrated on progesterone because in the state of hyperprogesteronemia in females, edema was virtually absent (Roof et al. 1993). Subsequent studies showed the protective effects of progesterone in males by examining both edema and cognitive recovery (Roof et al. 1994). Additionally, a recent study showed that progesterone at low but not high physiological levels protected against secondary injury to the hippocampus following brain injury (Robertson et al. 2006). It has also been shown that administration of physiological (Dubal and Wise 2001) or pharmacological (Simpkins et al. 1997) levels of estrogen following cerebral ischemia dramatically reduces ischemic volume. Indeed, in some studies it was shown that exogenous progesterone has a dose- and time-dependent neuroprotective action in experimental stroke (Murphy et al. 2002). The role of estrogens in traumatic brain injury has not been well illuminated, while a neuroprotective role for estrogens in cerebral ischemia has been suggested (Stein et al. 2008).

Cerebrovascular inflammation is an early event in the pathogenesis of stroke, and in secondary brain injury is believed to contribute by promoting leukocyte infiltration into brain (Frijns and Kappelle 2002), and blood–brain barrier (BBB) injury (del Zoppo and Hallenbeck 2000), and vasogenic edema (de Vries et al. 1997). The inflammation begins through the synthesis of cytokines, which signals the induction of proinflammatory mediators (Ospina et al. 2004). The disturbances of brain cytokines and growth factors including interleukin (IL)-1β and transforming growth factor-β (TGF-β) were implicated in the post-traumatic inflammatory cascade in earlier studies (Olsson 1995; de Vries et al. 1997; Hans et al. 1999).

Estrogens have a potent anti-inflammatory effect (Ospina et al. 2004) with regard to cerebral vascular response to IL-1β, while progesterone reduces cerebral edema by attenutating the production of proinflammatory cytokines (IL-1β, TNF-α) after TBI (He et al. 2004). An in vitro study also revealed that progesterone and estrogens reduce IL-β mRNA levels in monocytes (Polan et al. 1989).

The increased incidence of stroke and cerebral edema after menopause and in ovariectomized female animals is compatible with diminished circulating levels of estrogen and progesterone. While progesterone showed an anti-inflammatory effect, this effect is synergic when estrogens and progesterone are present together (Tibbetts et al. 1999). On the other hand, physiological levels of progesterone led to an increase in the inflammatory cytokines in multiple sclerosis (Hoffman et al. 2001; Koski et al. 2004), but when estrogen and progesterone were combined, the damage was limited (Hoffman et al. 2001) and progesterone receptor expression was up-regulated in the brain (Murphy et al. 2002). The present investigation was carried out to analyze brain edema and the intracerebral concentrations of proinflammatory cytokines (IL-β and TGF-β) in response to administration of progesterone and estrogen (physiological and pharmacological levels), alone and in combination, following diffuse traumatic brain injury in ovariectomized female rats.

Materials and methods

Animals

This study was conducted in accordance with the guidelines for the animal experimental protocols of Kerman University of Medical Sciences. The protocol was approved by the ethics committee (No EC/KNRC/86–30) of this university, in accordance with the internationally accepted principles for laboratory animal use and care, as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or US guidelines (NIH publication #85–23, revised in 1985). Animals (mature female Albino N Mary rats, weighing 200–250 g) were housed in an air-conditioned room at 22–25 °C, with a 12 h light : 12 h dark cycle and free access to food and water.

Method of bilateral ovariectomy

The animals were first anesthetized by injection of 60 mg/kg thiopental (intraperitoneal). The subabdominal area of the body was then shaved and an incision of 2 cm was made. The skin, fascia, and abdominal muscles were opened. Fats and intestine were sheered off until the uterus and its tubes were exposed. Catgut 4 thread was then twisted around the tube of the uterus and vascular base of the ovaries in the proximal area and cut from the distal area. We then poured 1–2 mL saline solution into the abdomen and the muscles and skin were replaced. The incision was stitched using 0–2 silk thread and the wound was washed with Betadine solution. To avoid interference due to the estrus cycle, all experimental animals were ovariectomized (OVX) 2 weeks before the experiments (Crandall et al. 2006).

Experimental protocols

Before the OVX animals were injured using the TBI technique, they were randomly divided into 9 groups, as follows: (*i*) Vehicle-treated group: OVX rats received an injection of an equal volume of vehicle (benzyl alcohol and sesame oil, which were used as estrogen or progesterone solvent), after TBI; (*ii*) E1-treated group: animals received an injection of a physiological dose of estrogen (33.3 μg/kg); (*iii*) E2-treated group: rats received an injection of a pharmacological dose of estrogen (1 mg/kg); (*iv*) P1-treated group: rats received an injection of a physiological dose of progesterone (1.7 mg/kg); (*v*) P2-treated group: rats received an injection of a pharmacological dose of progesterone (8 mg/kg); (*vi*) E1+P1-treated group: rats received an injection of combined physiological doses of estrogen and progesterone; (*vii*) E1+P2-treated group: rats received an injection of a combined physiological dose of estrogen and a pharmacological

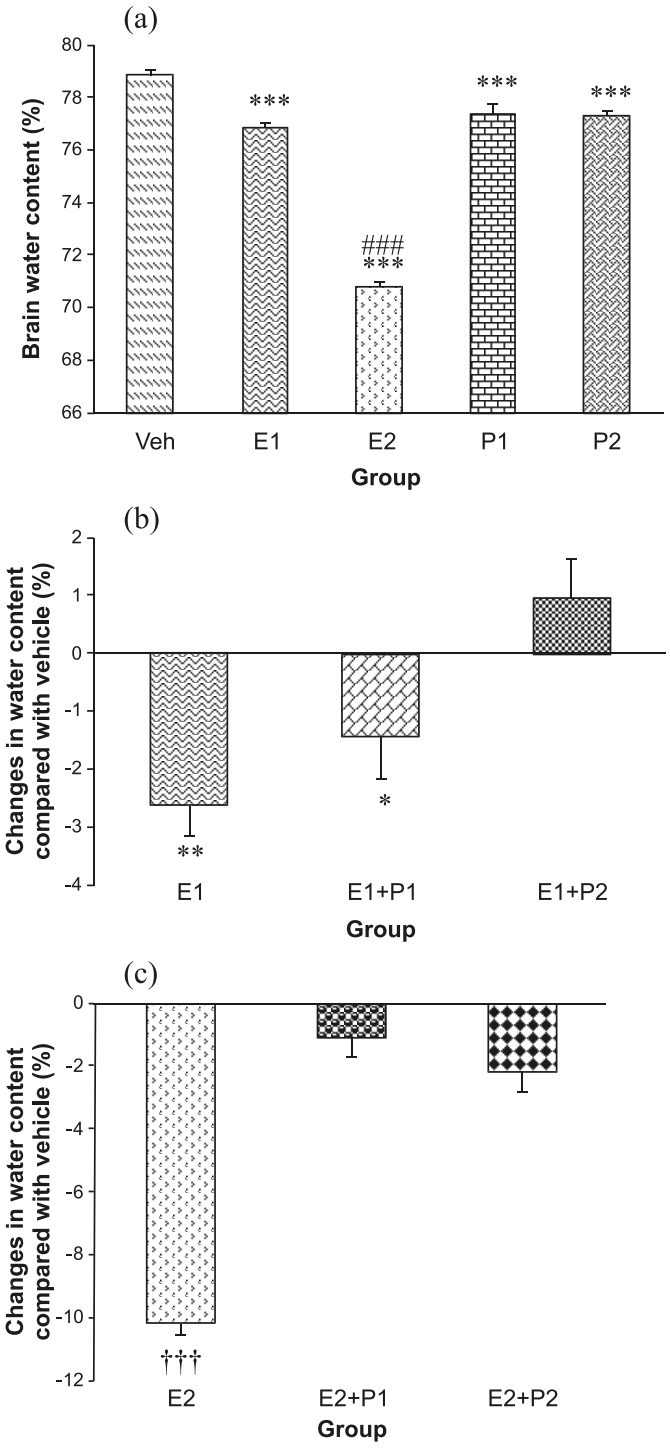

Fig. 1. (*a*) Brain water content (%) after traumatic brain injury in ovariectomized (OVX) rats ($n = 7$ in each group). ***, $p < 0.001$, all groups vs. Vehicle. ###, $p < 0.001$, E2 vs. all groups. (*b*) Changes in percentage of water content after TBI compared with vehicle in OVX rats ($n = 7$ in each group). **, $p < 0.01$, E1 vs. E1+P2 group. *, $p < 0.05$, E1+P1 vs. E1+P2 group. (*c*) Changes in percentage of water content after TBI compared with vehicle, in OVX rats ($n = 7$ in each group). †††, $p < 0.001$, E2 vs. E2+P1 or E2+P2 groups. Veh, vehicle; E1, physiological dose of estrogen; E2, pharmacological dose of estrogen; P1, physiological dose of progesterone; P2, pharmacological dose of progesterone; E1+P1, physiological dose of estrogen + physiological dose of progesterone; E1+P2, physiological dose of estrogen + pharmacological dose of progesterone; E2+P1, pharmacological dose of estrogen + physiological dose of progesterone; E2+P2, pharmacological dose of estrogen + pharmacological dose of progesterone. Data are presented as mean ± SEM.

dose of progesterone; (*viii*) E2+P1-treated group: rats received an injection of a combined pharmacological dose of estrogen and a physiological dose of progesterone; and (*ix*) E2+P2-treated group: rats received an injection of combined pharmacological doses of estrogen and progesterone.

It should be mentioned that the doses of estrogen and progesterone were given as a single intraperitoneal injection (i.p.), half an hour after TBI (O'Connor et al. 2005).

Model of diffuse traumatic brain injury (TBI)

All animals were intubated before TBI. The TBI method was moderate and of the diffuse type induced by the Marmarou method (O'Connor et al. 2005), using a TBI induction device made by the Dept. of Physiology, Kerman University of Medical Sciences. The protocol was as follows: a 250 g weight was dropped from a 2 m height onto the head of the anesthetized (with ether) rat while a metal disc (stainless steel, 10 mm in diameter, 3 mm thick) was attached to the animal's skull. After induction of the trauma, the rats were immediately connected to a respiratory pump (TSA animal respiratory compact, Germany). After spontaneous breathing had been restored, the intratracheal tube was removed and, following recovery, the rats were placed in individual cages.

Determination of brain water content

The brain edema of each animal was assessed by measuring brain water content. Anesthetized animals were sacrificed by cervical dislocation 24 h after TBI, the brain was removed, and brain samples were placed in pre-weighed glass vials and weighed (wet weight). The lids were removed and the vials placed in an incubator (Memmert, Germany) at 60 °C for 72 h, and then reweighed (dry weight). The percentage of water in each sample was then calculated using a formula published previously (Galani et al. 2001; O'Connor et al. 2005): (100 × [(wet weight–dry weight)/wet weight]).

Determination of blood–brain barrier (BBB) disruption

The degree of BBB disruption was assessed by measuring Evans blue dye leakage (O'Connor et al. 2005), with a slight modification. Briefly, Evans blue dye was dissolved in 0.01 mol/L PBS at a concentration of 2%, then the dye (2 mL/kg i.v.) was injected into the tail vein 4 h after TBI, as a BBB permeability tracer. The rats were then deeply anesthetized with ether and transcardiac perfused with 200 mL heparinized saline through the left ventricle to remove the intravascular dye. The brains were removed, then dissected, weighed, and stored at –80 °C for quantitative measurement. Brain samples were homogenized in 1 mL of 0.1 mol/L PBS, and 0.7 mL of 100% (*w*/*v*) trichloroacetic acid was added to it, and centrifuged. After centrifugation

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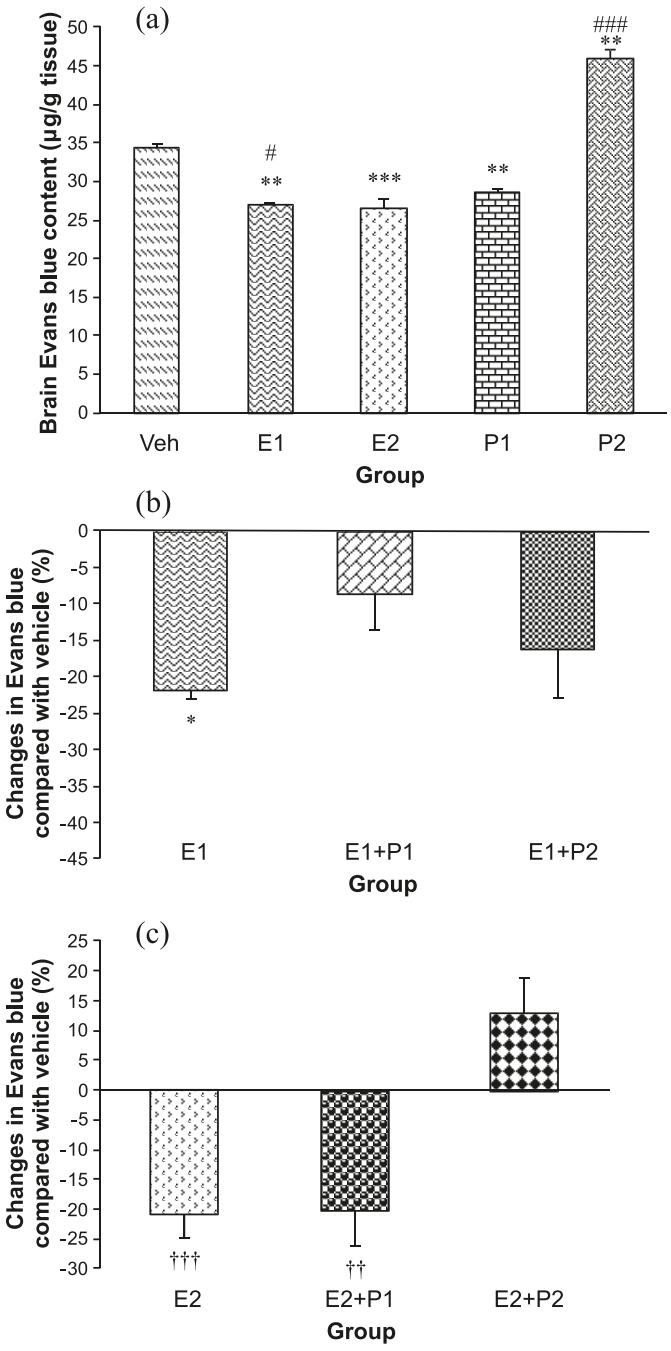

Fig. 2. (*a*) Quantitative measurement of Evans blue content (μg/g tissue) after traumatic brain injury (TBI) in ovariectomized (OVX) rats ($n = 7$ in each group). **, $p < 0.01$, E1, P1, and P2 groups vs. Vehicle. ***, $p < 0.001$, E2 group vs. Vehicle. #, $p < 0.05$, E1 vs. P1 group. ###, $p < 0.001$, P2 vs. all groups. (*b*) Changes in percentage of Evans blue content after TBI compared with vehicle, in OVX rats ($n = 7$ in each group). *, $p < 0.05$, E1 group vs. E1+P1. (*c*) Changes in percentage of Evans blue content after TBI compared with vehicle, in OVX rats ($n = 7$ in each group). †††, $p < 0.001$, E2 vs. E2+P2 group. ††, $p < 0.01$, E2+P1 vs. E2+P2 group. Veh, vehicle; E1, physiological dose of estrogen; E2, pharmacological dose of estrogen; P1, physiological dose of progesterone; P2, pharmacological dose of progesterone; E1+P1, physiological dose of estrogen + physiological dose of progesterone; E1+P2, physiological dose of estrogen + pharmacological dose of progesterone; E2+P1, pharmacological dose of estrogen + physiological dose of progesterone; E2+P2, pharmacological dose of estrogen + pharmacological dose of progesterone. Data are presented as mean ± SEM.

for 30 min at 1000*g*, the absorbance of Evans blue in supernatant was measured at 610 nm using a spectrophotometer (UV/VIS, Spectrometer, UK). The amount of extravasated Evans blue dye was quantified as μg/g brain tissue.

Measurement of brain cytokines and hormones

Rats were given an overdose of thiopental (50 mg/kg i.p.) 24 h after TBI (Dubal et al. 2001; Holmin and Höjeberg 2004) and perfused intracardially with PBS (pH, 7.4) for 1 min to remove the vesicular blood, which might have contained cytokines from the periphery. The brains were then quickly removed and immediately frozen in liquid nitrogen. The brains were weighed and homogenized in T-PER Tissue Protein Extraction Reagent (Taupin et al. 1993) with 0.5% Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris, and a protease inhibitor cocktail (Pierce) (500 mg tissue per 1 mL of the reagent). Following homogenization, the samples were shaken in a shaker for 90 min and then centrifuged (4 °C and 4000*g*) for 15 min. The homogenate supernatant was collected. The protein content of the supernatant was estimated using a BCA Protein Assay Reagent Kit to ensure that an equal amount of protein from each sample was used for the assay (Taupin et al. 1993). ELISA kits for IL-1β and TGF-β were purchased from Austrian BMS. Evaluation of cytokines was carried out following protocols provided by the manufacturer. The concentration of the cytokines was quantified as a picogram or nanogram of antigen per milligram of protein.

Statistical analysis

Quantitative data were expressed as mean ± SEM. The data were analyzed by parametric analysis of variance (ANOVA) or independent *t* test. One-way ANOVA was used for concentration analysis and an independent *t* test was used for a percent analysis of the changes. Fisher's LSD was employed for the ANOVA post-hoc analysis and Levene's Test for independent *t* test post-hoc analysis. The criterion for statistical significance was set at $p < 0.05$.

Results

Brain water content

Changes in the brain water content of the ovariectomized rats are shown in Fig. 1. Fig. 1*a* shows that the water content in the groups treated with high (E2) and low (E1) doses of estrogen and high (P2) and low (P1) doses of progesterone was significantly lower than that in the vehicle-treated group (78.84% ± 0.27, $p < 0.001$). In addition, the water content in the E2 (70.8% ± 0.19)-treated group was statistically different from the other treated groups ($p < 0.001$). Fig. 1*b* shows the effect of a low dose of estrogen on brain water content compared with the vehicle-treated group. A low dose of estrogen decreased brain water content

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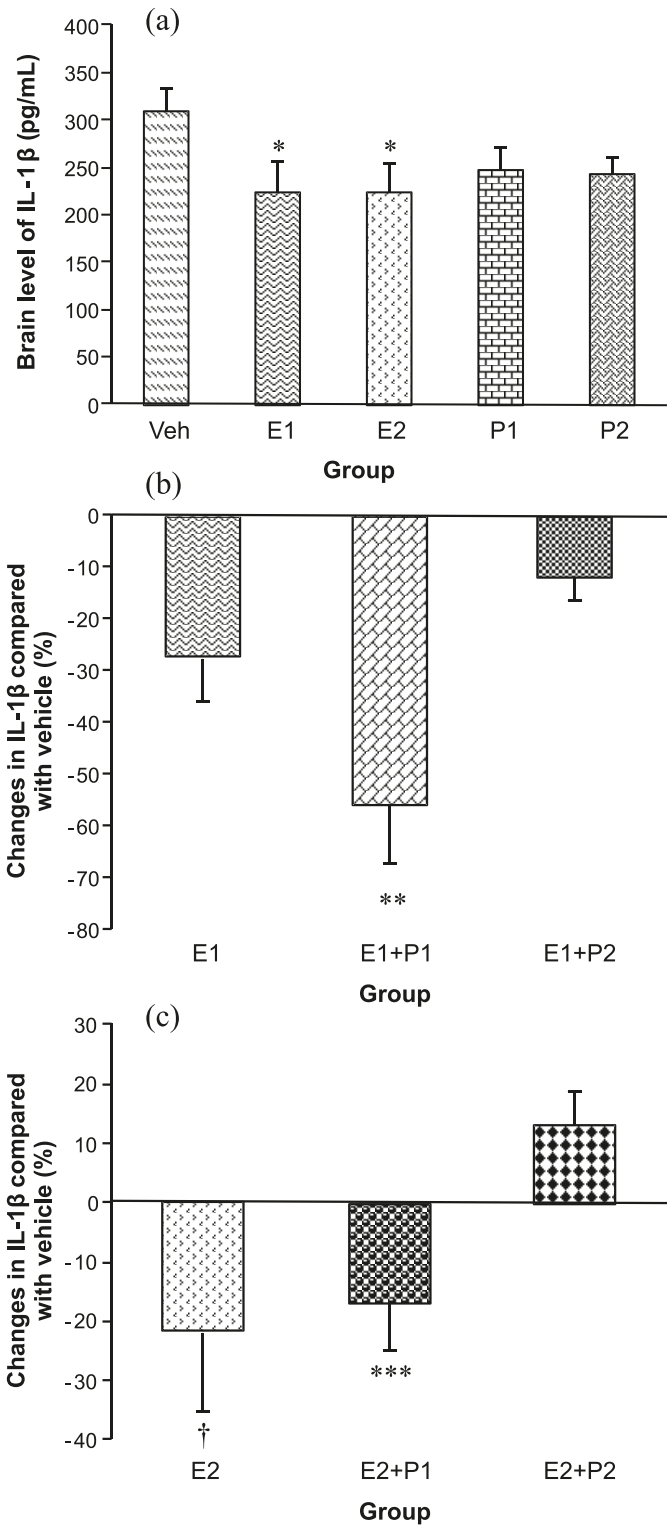

Fig. 3. (*a*) The effect of estrogen or progesterone following traumatic brain injury (TBI) on brain levels of IL-1β (pg/mL) in ovariectomized (OVX) rats ($n = 7$ in each group). *, $p < 0.05$, E1 or E2 groups vs. Vehicle. (*b*) Changes in the percentage of brain level of IL-1β after TBI compared with the vehicle, in OVX rats ($n = 7$ in each group). **, $p < 0.01$, E1+P1 vs. E1+P2 group. (*c*) Changes in the percentage of brain level of IL-1β after TBI compared with the vehicle, in OVX rats ($n = 7$ in each group). ***, $p < 0.001$, E2+P1 vs. E2+P2 group. †, $p < 0.05$, E2 vs. E2+P2 group. Veh, vehicle; E1, physiological dose of estrogen; E2, pharmacological dose of estrogen; P1, physiological dose of progesterone; P2, pharmacological dose of progesterone; E1+P1, physiological dose of estrogen + physiological dose of progesterone; E1+P2, physiological dose of estrogen + pharmacological dose of progesterone; E2+P1, pharmacological dose of estrogen + physiological dose of progesterone; E2+P2, pharmacological dose of estrogen + pharmacological dose of progesterone. Data are presented as mean ± SEM.

(–2.63% ± 0.5) compared with the E1+P2 (0.97% ± 0.68, $p < 0.01$)-treated group. The effect of the E1+P2 treatment on water content was also statistically different from that of the E1+P1 group (–1.42% ± 0.75, $p < 0.05$). Changes in brain water content in the group treated with a high dose of estrogen compared with the vehicle-treated group are shown in Fig. 1*c*. Water content in the E2 (–10.19% ± 0.37)-treated group was significantly less than in the E2+P1 (–1.06 ± 0.63, $p < 0.001$) and E2+P2 (–2.15 ± 0.69, $p < 0.001$)-treated groups.

Extravasation of Evans blue content

The effect of different doses of ovarian steroid hormones on BBB disruption after TBI is shown in Fig. 2. Fig. 2*a* shows that the Evans blue content in the E1 (27.03 ± 0.32 μg/g tissue, $p < 0.001$), E2 (26.7 ± 1.09 μg/g tissue, $p < 0.01$), and P1 (28.6 ± 0.52 μg/g tissue, $p < 0.01$)-treated groups was significantly lower than that of the vehicle-treated group, while the Evans blue content in the P2 (46.05 ± 1.06 μg/g tissue, $p < 0.01$)-treated group was significantly higher than in the vehicle-treated group. The P2 group increased its Evans blue content, compared with all the other groups ($p < 0.001$). The Evans blue content in the E1 group was also lower than in the P1 group ($p < 0.05$). Fig. 2*b* shows the effect of a low dose of estrogen on Evans blue content compared with the vehicle-treated group. The E1 group showed a significant decrease in Evans blue content (–21.85% ± 1.23) compared with the E1+P1 (–8.57% ± 4.49)-treated group ($p < 0.05$). The change in the Evans blue content in the group treated with a high dose of estrogen compared with the vehicle-treated group is shown in Fig. 2*c*. The level of Evans blue decreased in the E2 (–20.81% ± 3.87)-treated group, while this level increased in the E2+P2 (13.17% ± 5.83)-treated group. There was a significant difference in the level of Evans blue content between the E2+P2-treated group and the E2 ($p < 0.001$) and E2+P1 ($p < 0.01$)-treated groups, as well.

Brain level of IL-1β following treatment

The effect of different doses of ovarian steroid hormones on the brain level of IL-1β after TBI is shown in Fig. 3. Fig. 3*a* shows that the injured animals treated with either E1 (224.62 ± 32.8 pg/mL) or E2 (223.6 ± 30.1 pg/mL) had a lower level of IL-1β compared with the vehicle-treated animals ($p < 0.05$). Fig. 3*b* shows the effect of a low dose of estrogen on the brain IL-1β level compared with that in the vehicle-treated group. The inhibitory effect of E1+P1 (–55.64% ± 11.44) treatment on IL-1β level was statistically higher than in the E1+P2 (–11.93% ± 3.94)-treated group ($p < 0.01$). The change in the brain level of IL-1β

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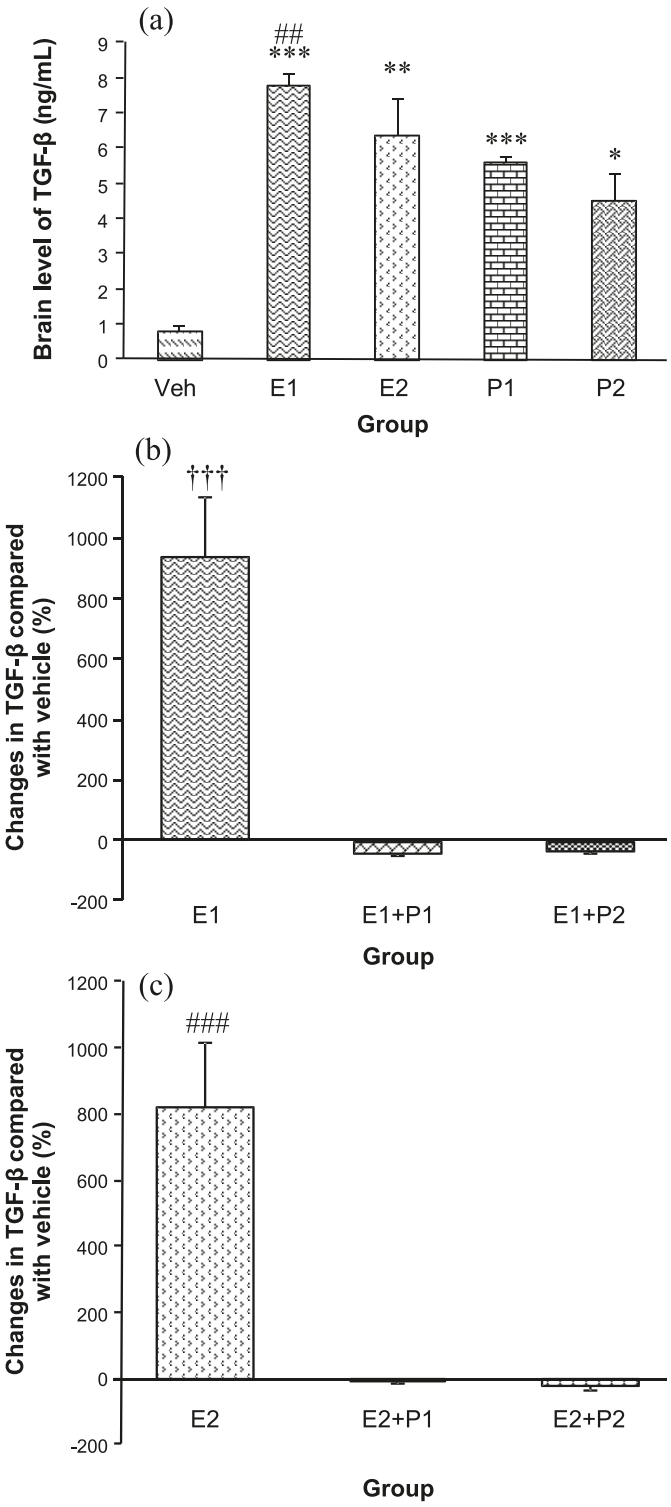

Fig. 4. (*a*) Effect of estrogen or progesterone following traumatic brain injury (TBI) on brain level of TGF-β (ng/mL) in ovariectomized (OVX) rats (n = 7 in each group). *, $p < 0.05$, P2 group vs. vehicle. **, $p < 0.01$, E2 group vs. vehicle. ***, $p < 0.001$, E1 and P1 groups vs. vehicle. ##, $p < 0.01$, E1 vs. P1 group. (*b*) Changes in the percentage of brain level of TGF-β after TBI compared with vehicle, in OVX rats (n = 7 in each group). †††, $p < 0.001$, E1 vs. E1+P1 or E1+P2 groups. (*c*) Changes in the percentage of brain level of TGF-β after TBI compared with the vehicle, in OVX rats (n = 7 in each group). ###, $p < 0.001$, E2 vs. E2+P1 or E2+P2 groups. Veh, vehicle; E1, physiological dose of estrogen; E2, pharmacological dose of estrogen; P1, physiological dose of progesterone; P2, pharmacological dose of progesterone; E1+P1, physiological dose of estrogen + physiological dose of progesterone; E1+P2, physiological dose of estrogen + pharmacological dose of progesterone; E2+P1, pharmacological dose of estrogen + physiological dose of progesterone; E2+P2, pharmacological dose of

in the group treated with a high dose of estrogen compared with the vehicle-treated group is shown in Fig. 3*c*. The inhibitory effect of E2 on IL-1β (–21.56% ± 13.6) was higher than in the E2+P2 (13.35% ± 5.84)-treated group ($p < 0.05$). There was also a statistically significant difference between the E2+P1 (–16.9% ± 7.98)-treated group and the E2+P2-treated group ($p < 0.001$).

Brain level of TGF-β following treatment

The effect of different doses of ovarian steroid hormones on the brain level of TGF-β after TBI is shown in Fig. 4. Fig. 4*a* shows that in both the E1 (7.8 ±0.3 ng/mL) and P1 (5.6 ± 0.2 ng/mL)-treated groups, the brain level of TGF-β significantly increased compared with the vehicle-treated group (0.8 ± 0.2 ng/mL, $p < 0.001$). There were also statistical differences between the E2 (6.4 ± 1 ng/mL, $p < 0.01$) and P2 (4.5 ± 0.8 ng/mL, $p < 0.05$)-treated groups compared with the vehicle-treated group. The TGF-β level in the E1 group was also significantly higher than in the P1 group ($p < 0.01$).

The change in the brain level of TGF-β in the group treated with a low dose of estrogen compared with the vehicle group is shown in Fig. 4*b*. The TGF-β level in the E1-treated group significantly increased (937.71% ± 202.9) compared with the E1+P1 (–37.99% ± 6.26) and E1+P2 (–32.27% ± 5.05)-treated groups ($p < 0.001$). Fig. 4*c* shows the effect of a high dose of estrogen on the brain level of TGF-β compared with the vehicle-treated group. Changes in TGF-β in the E2-treated group (821.72% ± 193.94) were also significantly higher than in the E2+P1 (–6.92% ± 3.67) and E2+P2 (–19.15% ± 16.68)-treated groups ($p < 0.001$). The TGF-β level in the case of a combined administration of estrogen and progesterone shows a decrease, instead of an increase.

Alternation in brain levels of sex steroid hormones

Table 1 shows brain levels of 17β-estrogen and progesterone 24 h after TBI. The brain level of 17β-estrogen in the E2 (608.7 ± 94.3 pg/mL) and E2+P1 (568.1 ± 53.8 pg/mL)-treated groups was higher than in the vehicle-treated group (333.4 ± 10.6 pg/mL, $p < 0.001$). There was also a significant difference ($p < 0.001$) between the E2+P1 and E1+P2 (367.7 ± 10.7 pg/mL)-treated groups.

The brain level of progesterone showed a significant difference ($p < 0.01$) in the P2 (27.7 ± 3.5 ng/mL) compared with the vehicle-treated groups (12.3 ± 0.5 ng/mL).

Discussion

Secondary trauma cascades are activated after TBI. They include severe brain inflammatory responses, which play a

Table 1. Change in brain concentrations of 17β-estradiol and progesterone in different groups of study, 24 h after traumatic brain injury (TBI).

Hormone	Group								
	Veh	E1	E2	P1	P2	E1+P1	E1+P2	E2+P1	E2+P2
17β-stradiol (pg/ml)	333.4±10.6	365±5.2	608.7±94.3[a]	339.9±14.9	336±11.6	451.2±64.05	367.7±10.7	568.1±53.8[b]	487.5±36.7
progesterone (ng/mL)	12.3±0.5	15.3±4.3	20.5±2.1	14.8±1.6	27.7±3.5[c]	15.87±1.06	17.68±2.26	16.42±1.84	19.7±2.3

Note: Veh, vehicle; E1, physiological dose of estrogen; E2, pharmacological dose of estrogen; P1, physiological dose of progesterone; P2, pharmacological dose of progesterone; E1+P1, physiological dose of estrogen + physiological dose of progesterone; E1+P2, physiological dose of estrogen + pharmacological dose of progesterone; E2+P1, pharmacological dose of estrogen + physiological dose of progesterone; E2+P2, pharmacological dose of estrogen + pharmacological dose of progesterone. Data are presented as mean ± SEM (n = 7 in each group).

[a]Significant difference between the E2-treated group and the vehicle group ($p < 0.001$).

[b]Significant difference between the E2+P1-treated group and the vehicle or E1+P2-treated groups ($p < 0.001$).

[c]Significant difference between the P2-treated group and the vehicle group ($p < 0.01$).

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role in the pathology of traumatic injury. BBB disruption, edema formation, activation of resident glial cells, and the release of inflammatory cytokines also form part of delayed responses to traumatic injury (de Vries et al. 1997; del Zoppo and Hallenbeck 2000). The present study shows that progesterone and estrogen administered alone and in combination cause a reduction in brain edema as well as an inhibition of BBB damage in TBI. The inflammatory effect may be mediated by affecting the brain levels of cytokines.

Both the therapeutic low dose (physiological dose) and the high dose (pharmacological dose) of estrogen lead to a reduction in water and Evans blue content. On the other hand, both doses of progesterone have an inhibitory effect on water content, while only the physiological dose of progesterone was able to inhibit Evans blue content. The greatest inhibitory effect was observed with the pharmacological dose of estrogen, which reduced 10.2% and 24.2% of the water content and the Evans blue content, respectively.

The inhibitory effect of estrogen on water content was abolished when a low dose of estrogen was used along with a high dose of progesterone (E1+P2), while the combination of E1+P1 did not show any changes. Co-administration of E2+P1 or E2+P2 led to a reduction of the inhibitory effect of estrogen. The inhibitory effect of estrogen on Evans blue content was reduced when E1+P1 was used, but any changes in the inhibitory effect of estrogen were not seen in the E1+P2 group. These results indicated that the inhibitory effect was not only abolished, but also inverted when high doses of estrogen and progesterone were used together (E2+P2). In addition, this study suggests that the effect of progesterone on water and Evans blue content is dose dependent. We also showed a significant difference in the inhibitory effect between the E1+P1- and E1+P2-treated groups for water content and between the E2+P1- and E2+P2-treated groups for Evans blue.

There are several probable mechanisms by which sex steroids could reduce the formation of brain edema that result from inflammatory response. These include as scavengers of free radicals (Gibson et al. 2005; Hoffman et al. 2006), improvement of cell viability and reduction of edema formation (Roof et al. 1997), binding to $GABA_A$ receptors, and modulating their functions (Baulieu et al. 1996). In addition, these hormones might decrease brain edema by acting on the BBB. The useful effects of steroids are not only limited to the nerve cells but also expand to the endothelial cells. Since the endothelium is one of the most important parts of the BBB (Razandi et al. 2000), the protective effects of steroids on the endothelial cells may enable them to protect the BBB against TBI. Estrogen reduces the damage to the BBB by inhibiting the expression of matrix metalloproteinase-2 (MMP-2), MMP-9, cyclooxygenase (Liu et al. 2005), and vascular endothelial growth factor (VEGF) effect, after brain ischemia (Chi et al. 2004). Progesterone also reduces brain edema by decreasing the permeability of the BBB (Gibson et al. 2005).

The effect of the E2+P1 treatment on the reduction of Evans blue content might be related to the elevation in brain estrogen level (only in this group, compared with the vehicle), because water and Evans blue content were reduced in all groups that administered progesterone and estrogen alone, except in the P2-treated group. Furthermore, the greatest effect of the pharmacological dose of estrogen compared with the other 3 therapeutic groups and the vehicle might be related to the highest brain level of estrogen.

With a pharmacological dose of progesterone, which shows a high level of this steroid hormone within the brain, Evans blue content increased, which means the low dose of progesterone inhibits Evans blue content, whereas its high dose increases it.

E1+P1 treatment caused the brain edema to decrease without decreasing the BBB; this finding is consistent with other studies that have reported that sex steroids can reduce brain edema while the BBB is intact (Betz and Coester 1990), or that these steroids reduce brain edema by the other mechanisms, such as the antioxidant mechanism (Gibson et al. 2005; Hoffman et al. 2006). The present results show the inhibition of water content (decreasing of the brain edema) following administration of progesterone and estrogen alone, but that the water content elevates following administration of the hormones combined. Furthermore, if a decrease in BBB disruption is desired, the administration of either progesterone or estrogen and a combination of the hormones, such as E1+P2 and E2+P1, could be helpful.

Our study also showed that a brain level of IL-1β is increased following TBI (data not shown). This finding is confirmed by previous studies (Lenzlinger et al. 2001; Kamm et al. 2006; Chen et al. 2008). In addition, our results showed

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that both doses of estrogen could reduce (27%) the level of IL-1β. This reducing effect of estrogen on the level of IL-1β is associated with a decrease in gene expression and maximum production of the cytokines (Hans et al. 1999).

In addition, nervous damage resulting from TBI is inhibited by controlled production of IL-1β (Lu et al. 2007). Administration of IL-1β antagonisms after TBI leads to a reduction of nervous inflammation; therefore, estrogen-induced changes in the brain level of IL-1β could suggest a mechanism that mediates anti-inflammatory and other neuroprotective effects. The lowering effects of estrogen on the level of IL-1β have been confirmed by other investigators, too (Yuan et al. 2002; Houdeau et al. 2007). It has also been confirmed by a number of studies, which reported that progesterone has no effect on the level of IL-1β (Holmin and Höjeberg 2004; Gibson et al. 2005; Jönsson 2007); therefore, the anti-inflammatory effect of progesterone should be performed by the other mechanisms (Lenzlinger et al. 2001; Gibson et al. 2005; Hoffman et al. 2006).

On the other hand, it has also been reported that progesterone causes a reduction in IL-1β (He et al. 2004; Gibson et al. 2005; Chen et al. 2008) and the increasing effect of estrogen on the level of IL-1β following TBI (Cutolo et al. 2006) is not similar to the result of the current study. The discrepancy between our results and previously reported data could be due to various factors, such as type of injury, severity, dose and time of administration of hormones, and type of solvent used. In addition, the combination of low doses of estrogen and progesterone (the E1+P1-treated group) could decrease the level of IL-1β more than the E1-treated group, but statistically, the differences were not significant, while the difference between this group (E1+P1) when compared with the value obtained for the E1+P2-treated group became significant. This means that the co-administration of a high dose of progesterone and a low dose of estrogen (E1+P2) leads to a decrease in the inhibitory effect of estrogen on IL-1β. A significant effect on IL-1β production was observed when high doses of estrogen and progesterone (E2+P2) were administered. These data correlated well with the results obtained from the Evans blue and water contents. These data could suggest that the reduction of IL-1β in the E1+P1- and E2+P1-treated groups, and less so in the E1+P2-treated group, might be one of the mechanisms that reduced brain edema by a combination of estrogen and progesterone. Furthermore, our data showed that the differences between the E1+P1- and E1+P2-treated groups, or between the E2+P1 and E2+P2-treated groups, were statistically significant. It can be concluded that the reducing effects of the combined groups on the IL-1β level is dose dependent, like the effects observed on the water and Evans blue contents. Previously, it was reported that the neuroprotective effects of progesterone on experimental stroke are dose and time dependent, while a physiological (low) dose of progesterone plays a protective role in ischemic female rats (Murphy et al. 2002) and decreases edema (Hoffman et al. 2006), but its high dose has no effect. Progesterone in the absence of estrogen led to an elevation of inflammation, but when used with estrogen, it had a powerful anti-inflammatory effect (Liu et al. 2005) by reducing the IL-1β level (Yuan et al. 2002), or inhibiting IL-1β action (Schaefer et al. 2005).

The present study indicates that both doses of estrogen or progesterone cause the level of TGF-β to increase following TBI, but the estrogen has a more powerful effect than that of progesterone. Previous studies indicated that the anti-inflammatory actions of TGF-β are dominant and, as the anti-inflammatory effect of TGF-β is probably mediated by controlled production of IL-1β, TNF-α, and free radicals of oxygen (Chiaretti et al. 2008). The increasing TGF-β within the brain might be one of the mechanisms of the anti-inflammatory effect of steroids, as reported previously (Hatthachote and Gillespie 1999; Gibson et al. 2005). The hormones increase the level of TGF-β by changing the level of IL-1β (Hoffman et al. 2006) and releasing the amount of cytokines from brain and blood cells, and preventing damage to the BBB (Lenzlinger et al. 2001).

In the combined groups, co-administration of estrogen and progesterone leads to a reduction of their effects on the brain level of TGF-β. Most of these reductions were seen in the E1+P1- and E1+P2-treated groups. These data suggest that the effect is decreased or abolished when a high dose of estrogen is used in the combined groups. It is also revealed that the effects of progesterone in combined groups are not dose dependent. Based on the results of this part of the study, a co-administration of estrogen and progesterone is not recommended when the aim is increasing the level of TGF-β.

Overall, the present study showed that the administration of different doses of estrogen or progesterone alone (except the pharmacological dose of progesterone) leads to preventing BBB disruption following TBI. In addition to other neuroprotective mechanisms of estrogen, decreasing the brain level of IL-1β and increasing the level of TGF-β can be considered one of the mechanisms of the anti-inflammatory effects of different doses of estrogen. Progesterone also has an anti-inflammatory effect, which is probably mediated by increasing TGF-β, besides its other mechanisms. Our results also showed that co-administration of estrogen and a pharmacological dose of progesterone can decrease the neuroprotective effects of estrogen, while such combination does have an inhibitory effect on brain edema. The inhibitory effects of the combined groups on brain edema are consistent with their inhibitory effects on increasing the level of TGF-β and reducing the level of IL-1β. It is necessary to follow up this study with an investigation of which type of mechanisms and what receptors are mediated in the interaction between sex steroids and the cytokines.

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